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Melanosome Maturation Defect in Rab38-deficient Retinal Pigment Epithelium Results in Instability of Immature Melanosomes during Transient Melanogenesis

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Pathways of melanosome biogenesis in retinal pigment epithelial (RPE) cells have received less attention than those of skin melanocytes. Although the bulk of melanin synthesis in RPE cells occurs embryonically, it is not clear whether adult RPE cells continue to produce melanosomes. Here, we show that progression from pmel17-positive premelanosomes to tyrosinase-positive mature melanosomes in the RPE is largely complete before birth. Loss of functional Rab38 in the "chocolate" (*cht*) mouse causes dramatically reduced numbers of melanosomes in adult RPE, in contrast to the mild phenotype previously shown in skin melanocytes. Choroidal melanocytes in *cht* mice also have reduced melanosome numbers, but a continuing low level of melanosome biogenesis gradually overcomes the defect, unlike in the RPE. Partial compensation by Rab32 that occurs in skin melanocytes is less effective in the RPE, presumably because of the short time window for melanosome biogenesis. In *cht* RPE, premelanosomes form but delivery of tyrosinase is impaired. Premelanosomes that fail to deposit melanin are unstable in both *cht* and tyrosinase-deficient RPE. Together with the high levels of cathepsin D in immature melanosomes of the RPE, our results suggest that melanin deposition may protect the maturing melanosome from the activity of lumenal acid hydrolases.

INTRODUCTION

Melanosomes are found in melanocytes and in the cells of the retinal pigment epithelium (RPE). The RPE is a pigmented monolayer of cells that lies between the photoreceptors and the choriocapillaris, a layer of fenestrated capillaries that also contains many heavily pigmented melanocytes. Melanin within melanosomes of the RPE plays an important role in the routing of optic tracts during eye development (Jeffery, 1997). Although the function of melanosomes in the adult RPE and the melanocytes of the choroid is less clear, they are likely to play a role in protection from light and oxidation and to absorb light scatter (Schraermeyer and Heimann, 1999). The mammalian RPE does not normally turnover in the adult, and the majority of melanin synthesis in the RPE occurs during embryonic life (Carr and Siegel, 1979). There is controversy over whether there is a low level of melanin synthesis in the adult RPE (Schraermeyer, 1993). Nevertheless, the regulation of melanosome production in RPE cells clearly differs from that of melanocytes of the skin where melanosomes are constantly synthesized before being transferred to neighboring keratinocytes. Skin melanocytes readily make melanosomes in culture and cultured melanocyte cell lines have been used extensively to analyze pathways leading to mature melanosome biogenesis. Although immature melanosomes were originally thought to arise from the endoplasmic reticulum (Seiji and Iwashita, 1963), the work of Raposo and Marks has shown that multivesicular endosomes lie on the pathway of melanosome biogenesis (Berson *et al*., 2001; Raposo *et al*., 2001). Pmel17 is a transmembrane protein required for the generation of the striations within immature melanosomes upon which melanin is deposited. In melanocytes, pmel17 localizes to the internal vesicles of multivesicular endosomes/bodies (MVBs) and to vacuoles recognizable as immature (stage II) melanosomes due to the presence of striations (Raposo *et al*., 2001). Consistent with a role for MVBs in melanosome biogenesis, when pmel17 is expressed in nonmelanogenic cells the protein is targeted to the internal vesicles of MVBs where, if expressed at a high enough level, it induces the formation of striations (Berson *et al*., 2001). Melanin-synthesizing enzymes are delivered to the immature pmel17-containing melanosomes by a process involving both AP1 and AP3 (Theos *et al*., 2005), and melanin is deposited on the striations to form stage III (where both striations and melanin are visible) and stage IV melanosomes (where melanin deposition is complete and striations are obscured). Cultured RPE cells isolated from postnatal eyes do not readily make melanosomes, so pathways leading to melanosome biogenesis have not been analyzed in detail in the RPE.

In the present study, we have focused on the role of Rab38 in melanosome biogenesis in RPE cells. A recessive Gly19-to-Val mutation in Rab38 in the "chocolate" (*cht*) mouse (Loftus *et al*., 2002), a coat color mutant, prevents prenylation and results in an exclusively cytosolic protein (Wasmeier *et al*., 2006). The cytosolic protein is inactive and

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Abbreviations used: cht, chocolate; MVB, multivesicular endosome/body; RPE, retinal pigment epithelium.

unstable. Loss of functional Rab38 in the *cht* mouse causes a mild defect in coat color (Loftus *et al*., 2002). Depletion of the closely related Rab32 in *cht* melanocytes causes a dramatic loss of pigmentation, indicating that the near normal pigmentation in *cht* melanocytes results from functional compensation by Rab32 (Wasmeier *et al*., 2006). In melanocytes lacking Rab38 and Rab32, tyrosinase is degraded after exit from the *trans*-Golgi network (TGN), indicating that Rab38 and Rab32 regulate a step in the transport of melanogenic enzymes from the TGN to immature melanosomes (Wasmeier *et al*., 2006). The RPE provides a unique opportunity to analyze melanosome biogenesis in vivo during the short time window of intense melanogenesis. Unlike skin melanocytes, RPE cells and choroidal melanocytes retain their melanosomes after synthesis, making it possible to gain novel insight into the regulation of melanosome biogenesis and stability.

MATERIALS AND METHODS

Materials

Mouse anti-pmel17 (HMB45) was from DakoCytomation (Ely, Cambridgeshire, United Kingdom), sheep anti-TGN38 was from Serotec (Oxford, United Kingdom), and rabbit anti-mouse tyrosinase was a generous gift from M. Marks (University of Pennsylvania). Rabbit anti-Rab38 and Rab32 antibodies were generated as described previously (Wasmeier *et al*., 2006). Mouse anti-Rab27a (4B12) was described previously (Hume *et al*., 2001), and rabbit anti-calnexin was from Stressgen (Cambridge, United Kingdom). Intermediate rabbit anti-mouse antibodies were from DakoCytomation, and protein A gold was from University Medical Centre (Utrecht, The Netherlands).

Mice

C57BL/6J-*Rab38*^{cht} (+/*cht*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and they were maintained and propagated under United Kingdom project licenses 70/5071 and 70/6210 at the Central Biomedical Services of Imperial College London (Wasmeier *et al*., 2006). The outbred strain MF1was used as an albino (tyrosinase-deficient) strain.

Cells

Primary mouse melanocytes were isolated and cultured as described previously (Wasmeier *et al*., 2006), and primary mouse RPE cells, essentially free from other cell types, were isolated and cultured as described previously (Lopes *et al*., 2007). AtT20 mouse anterior pituitary cells were cultured in DMEM/F-12 (3:1 ratio) with 25 mM glucose, 15% fetal calf serum, and antibiotics.

Electron Microscopy (EM)

Conventional EM and cryoimmuno-EM was performed essentially as described previously (Futter *et al*., 2004). For eyes from postnatal day (P)8 mice and older, the cornea was cut off after fixation, and lens removed before further processing. For embryonic day (E)14 and P1 eyes, the cornea and lens were not removed. For cryoimmuno-EM, E14 eyes were embedded whole in gelatin, infiltrated with sucrose, and mounted on specimen carriers. P1 eyes were quartered before embedding, infiltration and mounting. For P8 and adult eyes, small pieces of eye cup were sandwiched between gelatin before infiltration and mounting. Specimens were viewed on a JEOL 1010 microscope (JEOL, Tokyo, Japan).

To quantitate the number of melanosomes/area of RPE consecutive EM images were taken from a continuous ribbon of RPE. The images were used to reconstitute the ribbon in Adobe Photoshop (Adobe Systems, Mountain View, CA). Stage II (striations but no melanin), III (striations and melanin), and IV (heavily melanized with no visible striations) were counted and divided by the ribbon area.

Quantification of pmel17 and tyrosinase-positive organelles and average gold particles/melanosome was performed on cryosections. Random images of RPE and choroid (whenever possible) of E14, P1, P8, and 4-mo-old (4m) animals were taken. The number of gold particles on each melanosome (both immature and mature) was counted ($n > 150$). The percentage of melanosomes positive for either marker was calculated, setting five gold particles/melanosome as the positive threshold. The mean number of gold particles/melanosome was calculated based on all melanosomes (whether positive or negative).

Western Blot Analysis

Isolated wild-type mouse RPE cells, neuroretina, and the remaining eyecup obtained during the process of RPE isolation were used. Samples were fractionated on 13% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were blocked in phosphate-buffered saline (PBS)/0.1% Tween 20 with 4% nonfat dried milk, incubated with primary antibody in PBS/Tween 20, washed four times in blocking solution, and then incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse, 1:5000; DakoCytomation) followed by washing in PBS/Tween 20. Bound antibody was detected using the ECL Plus Western Blotting detection system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

RESULTS

RPE Cells, but Not Choroidal Melanocytes, Are Severely Depigmented in the Absence of Functional Rab38

In contrast with skin melanocytes (Wasmeier *et al*., 2006), the RPE of 4m *cht/cht* mice exhibit a much reduced number of melanosomes, compared with wild-type controls (Figure 1). The few melanosomes that are present in the RPE are mature, and they show a normal distribution, clearly having access to the apical processes. The morphology of melanosome in homozygous *cht* RPE is near normal, although they are slightly less spindle-shaped than heterozygous controls (width/length ratio = 0.50 ± 0.14 for homozygous *cht* versus 0.46 ± 0.17 for heterozygous control). The heterozygous *cht* RPE shows a greatly increased number of melanosomes compared with homozygous *cht* RPE, although there is a small reduction in melanosome number compared with wild type (Figure 1). Conversely, homozygous *cht* choroidal melanocytes exhibit a much more subtle reduction in melanosome number versus wild-type controls (Figure 1).

Analysis of the RPE of 2-y-old (2y) mice shows that there are still very few melanosomes in the homozygous, compared with the heterozygous *cht* mice (Figure 1). In fact, the number of melanosomes in the RPE in 2y mice seems similar to the number at 4 mo, such that the differences between wild type, heterozygous and homozygous *cht* are retained. In contrast, the number of melanosomes/melanocyte in the choroidal melanocytes clearly increases in all mice, and by 2 y there is no clear difference in pigmentation between wild type, heterozygous and homozygous *cht*.

Rab38 and Rab32 Are Expressed in RPE Cells

Rab32 has been shown to partially compensate for Rab38 function in melanocytes of the skin (Wasmeier *et al*., 2006). To determine whether Rab32 is expressed in pigmented cells of the eye, we subjected protein extracts derived from primary cultures of RPE cells isolated from 3-wk-old wild-type mice, whole mouse eyes, eye cup (containing RPE and choroid), and retina (devoid of RPE and choroid) to immunoblot analysis using anti-Rab antibodies. Rab38 and Rab32 were present in primary RPE cells, in the eye, and eye cup, but they were undetectable in the neural retina (Figure 2).

The Majority of Melanosomes Are Synthesized before Birth in the RPE

It was not possible to determine the nature of the defect in melanosome biogenesis from the phenotype of the adult *cht/cht* RPE. Therefore, we examined the effect of loss of functional Rab38 on melanosome biogenesis at time points when melanin synthesis normally occurs in the RPE. This entailed examining mice of embryonic and early postnatal age. Therefore, heterozygous and homozygous *cht* mice were crossed so that heterozygous and homozygous *cht* littermates (readily distinguishable on the basis of coat color) could be compared. Although there seems to be a small reduction in melanosome number in heterozygous *cht* mice, there is still a clear difference between heterozygous and homozygous mice.

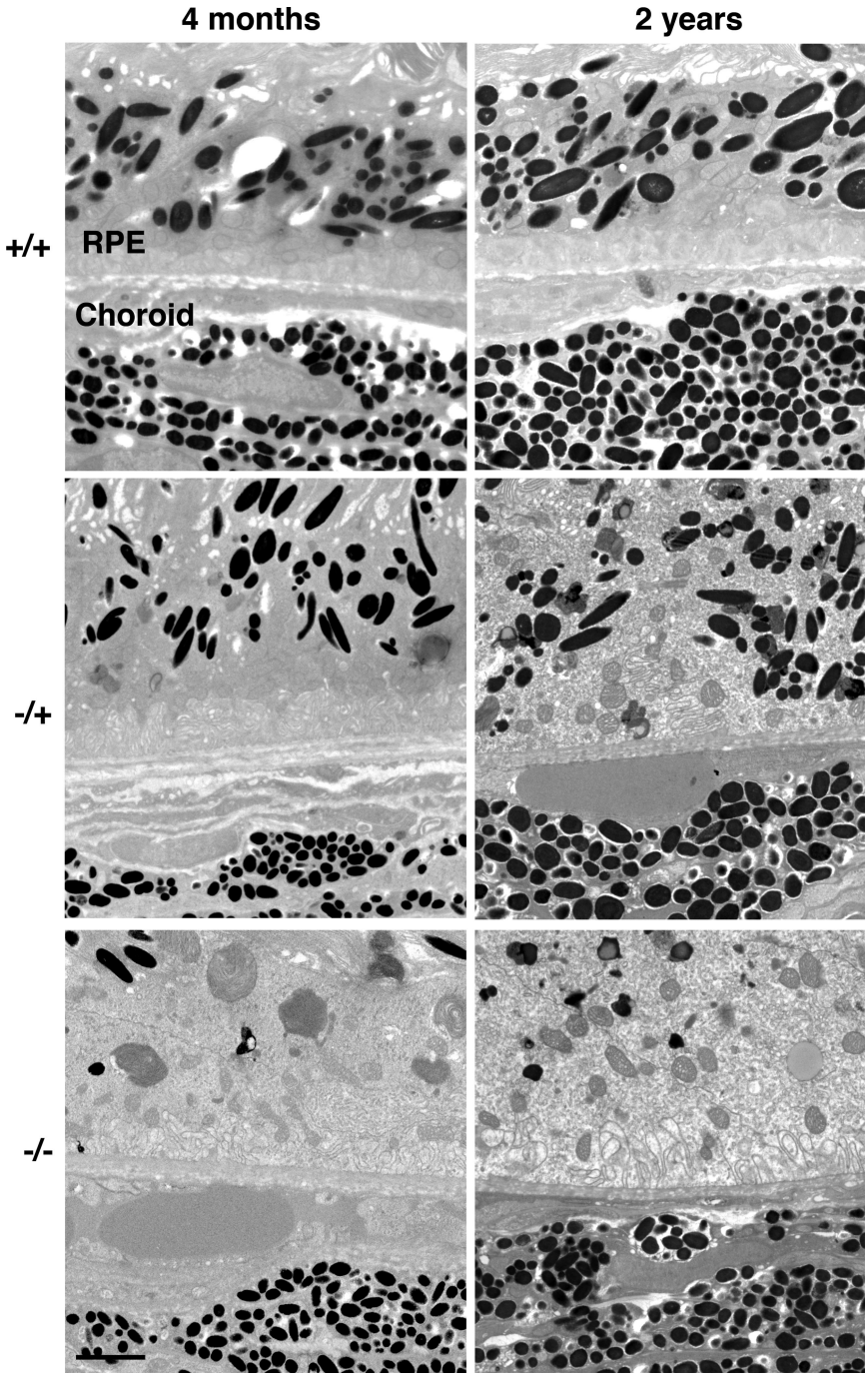


Figure 1. Pigmentation in the eye in wild-type and *cht* mice. Conventional EM of wild-type (+/+), heterozygous *cht* (−/+), and homozygous *cht* (−/−) RPE and choroid at 4 mo and 2 y, showing severe depigmentation in the *cht* RPE. Bar, 1.0 μm.

Melanosome biogenesis was first characterized in control (heterozygous) RPE. The majority of melanin synthesis occurs between E10 and E15 in mouse RPE (Bodenstein and Sidman, 1987; Beermann *et al*., 1992). Consistent with this timing, conventional EM showed both immature and mature melanosomes in the RPE of E14 control mice, but most melanosomes had apparently reached maturity (stage IV) by P1 (Figure 3). Although very few immature melanosomes could be observed at this age by conventional EM, cryoimmuno-EM showed that ~50% of the melanosomes at E14, P1, and P8 stained positive with anti-pmel17 antibody (HMB45) (Figures 3 and 4A for quantitation). This antibody has previously been found to stain predominantly immature melanosomes in melanocytes (Raposo *et al*., 2001). In the RPE, some apparently mature melanosomes stain positive with HMB45, but in those melanosomes the pmel17 staining is predominantly around the perimeter. The number of pmel17 gold particles/melanosome gradually declines from E14 to P8, suggesting either a gradual antigen masking or gradual removal of the protein (Figure 4B). In the adult (4m) RPE, pmel17 staining was virtually absent.

We also studied tyrosinase expression and localization in control mice at the same time points. At E14 and P1, there was strong staining of tyrosinase in the RPE, but this was considerably reduced by 8 d after birth and it was virtually undetectable in the adult (Figures 3 and 4A for quantitation).

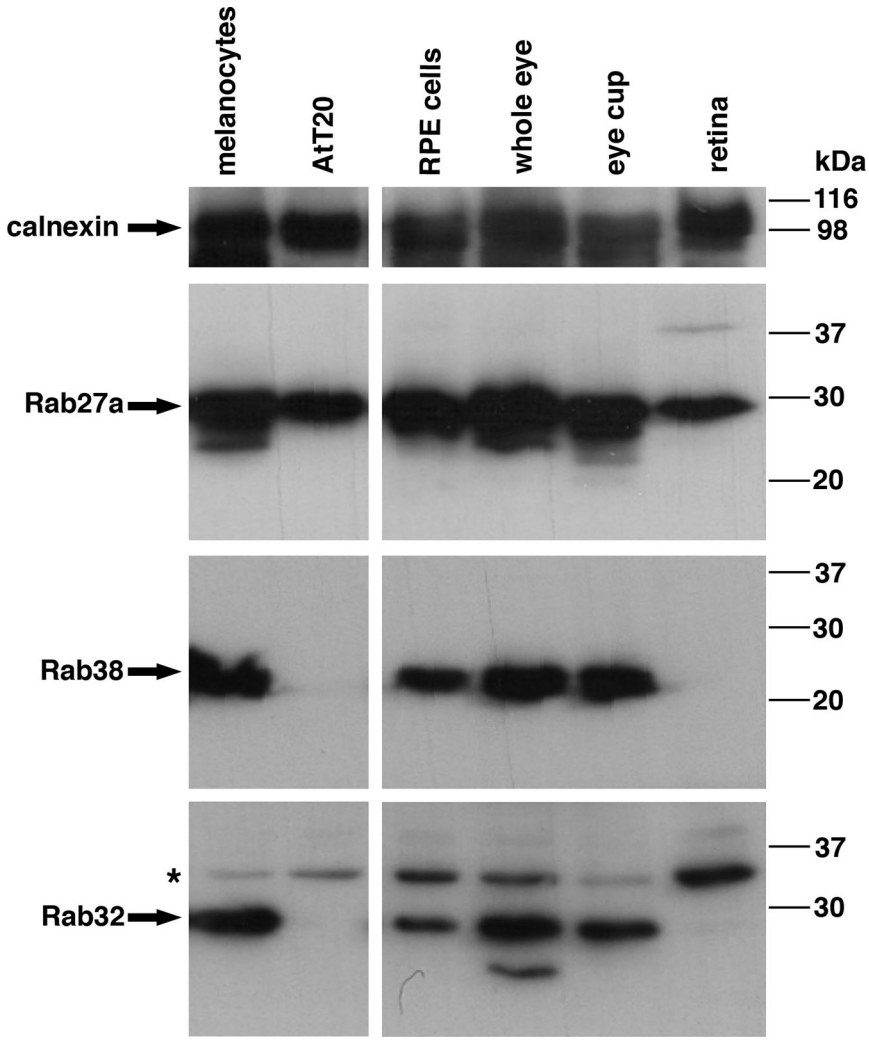


Figure 2. Ocular expression of Rab38 and Rab32. Proteins (25 μg/lane) from cultured cell lines (melanocytes or AtT20; left) or from different tissue preparations obtained from 3-wk-old wild-type mouse eyes (right) were separated by SDS-PAGE. Samples were analyzed by immunoblotting for Rab27a, Rab38, and Rab32 and for calnexin as a loading control. The asterisk indicates a nonspecific band in the Rab32 panels.

Interestingly, at E14 all melanin-containing melanosomes stained strongly for tyrosinase, whereas in 1- and 8-d-old mice, some melanosomes had largely lost tyrosinase staining. Quantitation confirmed that there was a decline in the percentage of tyrosinase positive melanosomes from E14 to P8 (Figure 4A), resulting in a decline in the mean number of tyrosinase gold particles/melanosome (Figure 4B). Overall, these results indicate that melanosome biogenesis occurs predominantly before birth in the RPE.

The Majority of Melanosomes Are Synthesized after Birth in Choroidal Melanocytes

Melanosome formation was also followed in choroidal melanocytes. Choroidal melanocytes could not be identified at E14, but both immature and mature melanosomes were present in choroidal melanocytes at P1 and P8 (Figure 4C). The number of pigmented melanocytes in the choroid at P1 was too low to allow quantitative cryoimmuno-EM, but at P8 the majority (>80%) of melanosomes stained positive for tyrosinase, and ~20% were positive for pmel17 (Figure 4A). Most mature melanosomes in choroidal melanocytes stained poorly for pmel17 (Figure 4C). This is in contrast to the RPE where some mature melanosomes stained for pmel17, but it is similar to skin melanocytes where pmel17 staining is largely confined to immature melanosomes (Raposo *et al.*, 2001). In the adult, the majority of melanosomes in choroidal melanocytes were mature, but occasional pmel17-positive vacuoles could be observed by cryoimmuno-EM (Figure 4, A and D). In many melanocytes of the adult choroid, tyrosinase staining was virtually undetectable, but in others it was present, although they had fewer tyrosinase gold particles than on melanosomes at early time points in either the RPE or choroidal melanocytes (Figure 4, B and D).

These results indicate that the majority of melanosomes are synthesized after birth in choroidal melanocytes. High levels of melanosome production continue for at least 8 d after birth, indicating a longer time window for the peak of melanosome synthesis than in the RPE. Our results are also consistent with the idea that a low level of melanosome synthesis is maintained in the adult choroid.

Premelanosomes Are Formed in the cht/cht RPE but Fail to Deposit Melanin

Conventional EM of the RPE of E14, 1- and 8-d-old *cht/cht* mice showed that the cytoplasm contained many striated vacuoles with the morphological appearance of immature (stage II) melanosomes with no melanin (Figure 5). Cryoimmuno-EM showed that these vacuoles stained strongly for pmel17, confirming their identity as immature melanosomes (Figure 5). In contrast, RPE from adult *cht/cht* mice contained

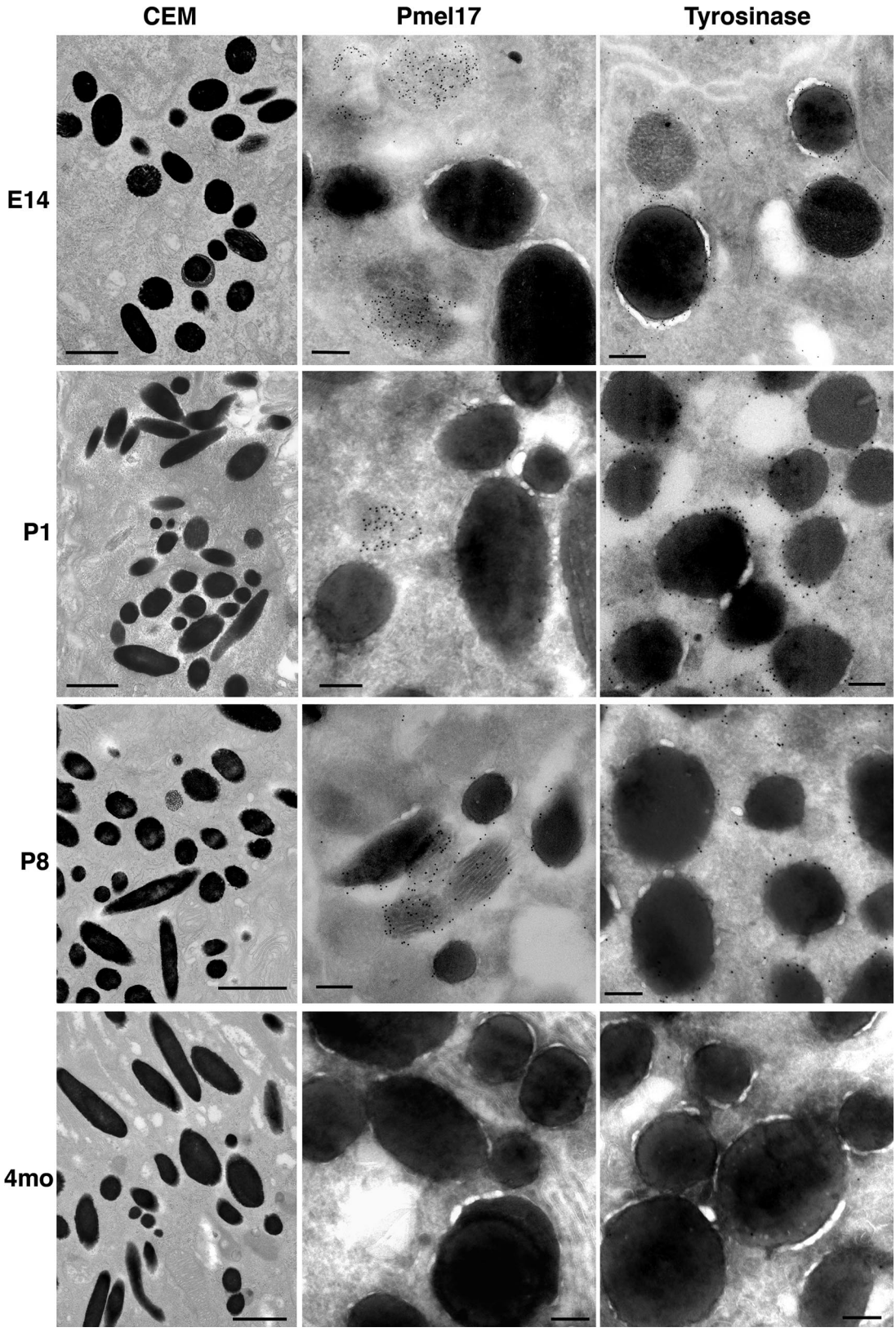


Figure 3. Melanosome biogenesis in RPE. Conventional EM (CEM) and cryoimmuno-EM showing pmel17 and tyrosinase staining of heterozygous *cht* RPE at E14, P1, P8, and 4 mo. Bars for conventional EM, 0.5 μm and for cryoimmuno-EM, 0.2 μm.

very few morphologically distinguishable immature melanosomes, and there was no detectable pmel17 staining (Figure 5). Quantitation of the numbers of melanosomes of different stages by conventional EM showed that in the

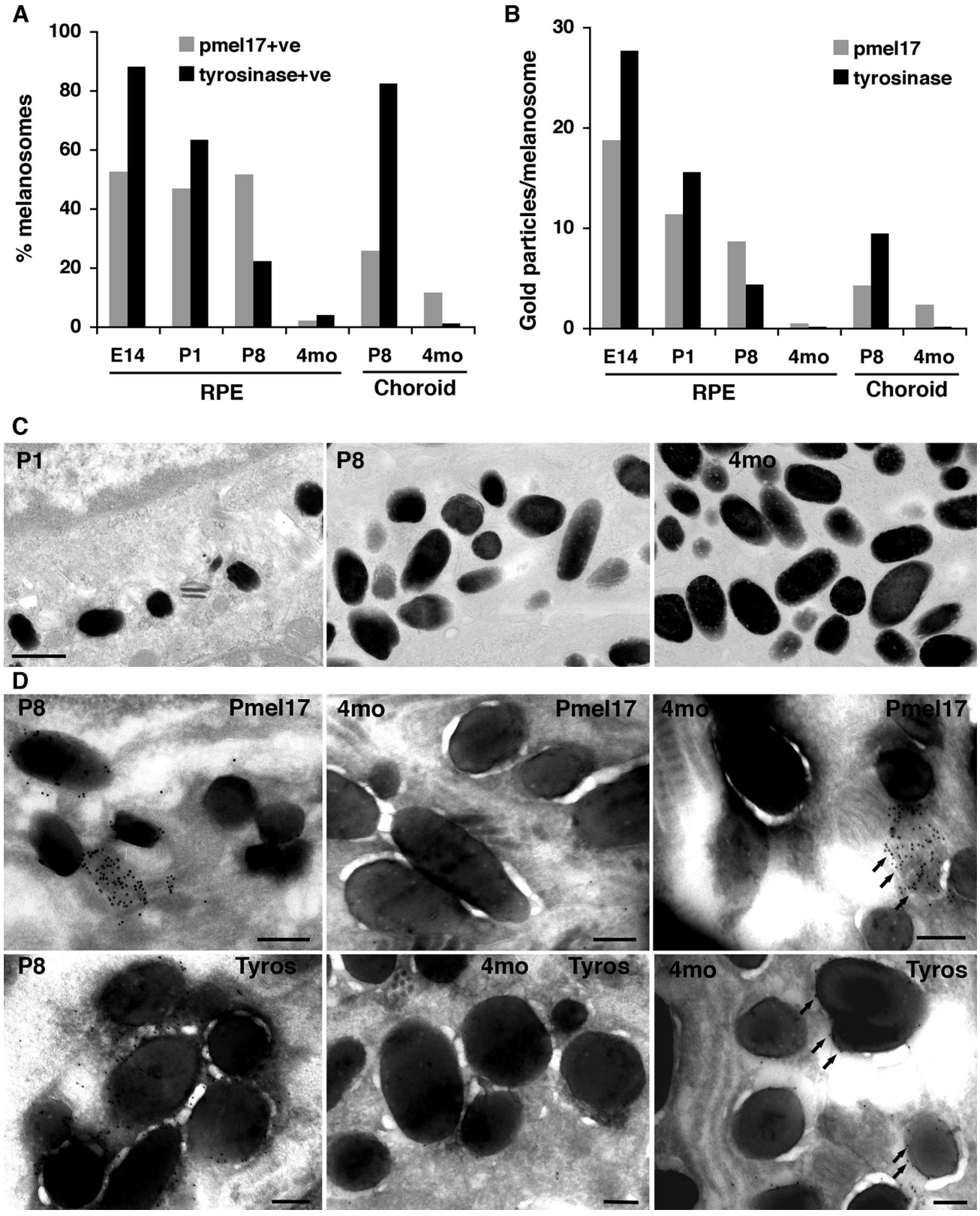


Figure 4. Melanosome biogenesis in choroidal melanocytes. The percentage of melanosomes (both immature and mature) in heterozygous *cht* RPE and choroidal melanocytes that were pmel17 positive and tyrosinase positive (at least 5 gold particles/melanosome) was quantitated (A), and the mean number of pmel17 and tyrosinase gold particles/melanosome (both immature and mature) was determined (B). Conventional EM (C) of P1, P8, and 4-mo-old heterozygous *cht* choroidal melanocytes and cryoimmuno-EM (D) showing pmel17 and tyrosinase staining (examples indicated by arrows) of heterozygous *cht* choroidal melanocytes at P8 and 4 mo old. Pigmented choroidal melanocytes at P1 were too rare to obtain cryoimmuno-EM data. Bars, 0.5 μm (C) and 0.2 μm (D).

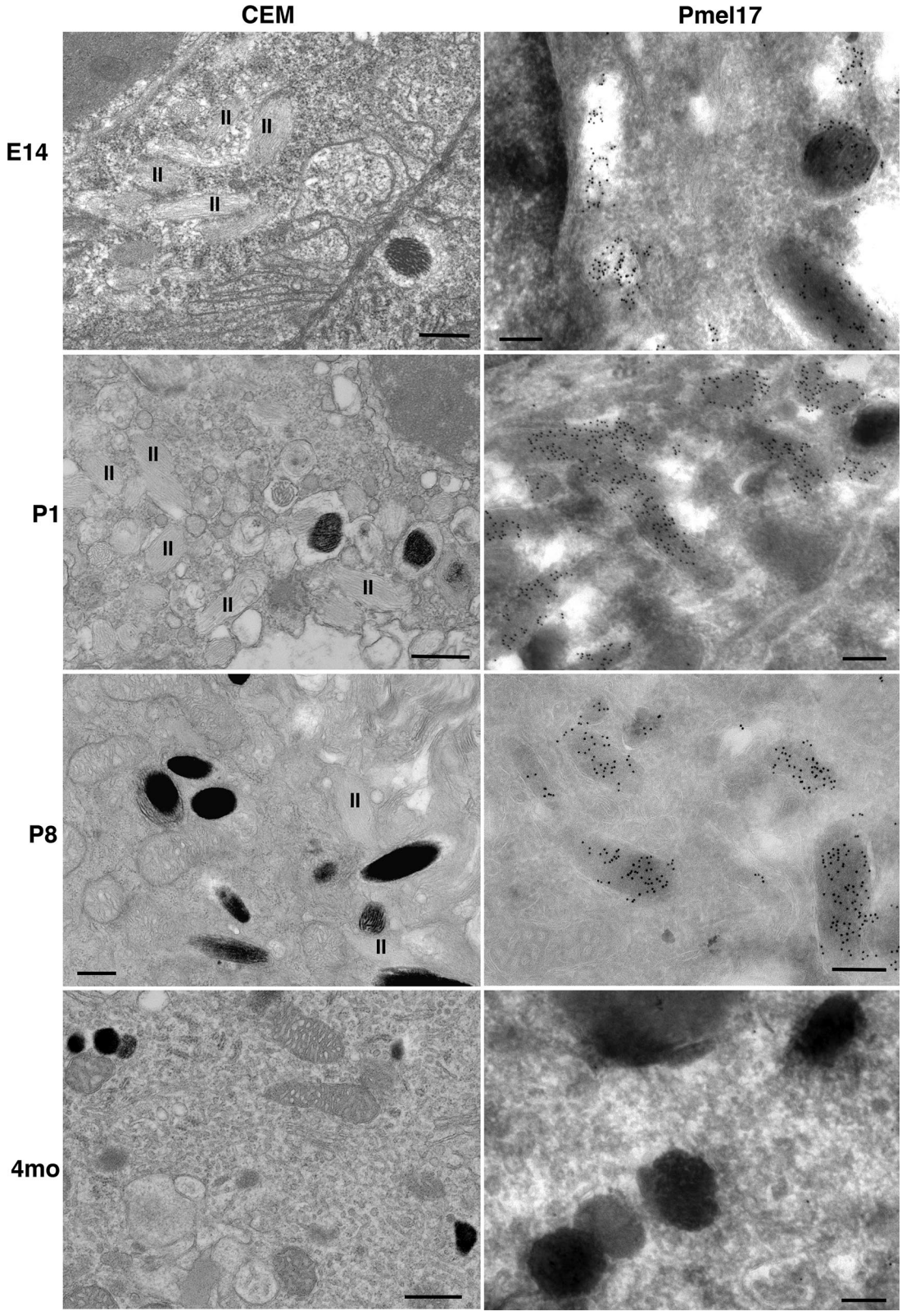


Figure 5. Premelanosome formation in *cht* RPE. CEM and cryoimmuno-EM showing pmel17 staining of homozygous *cht* RPE at E14, P1, P8, and 4 mo. Examples of stage II melanosomes are indicated (II). Bars for conventional EM, 0.5 μm and for cryoimmuno-EM, 0.2 μm.

cht/cht RPE at P1, the majority (60%) of melanosomes were stage II and only 13% were stage IV (Figure 6A). In contrast, 71% of melanosomes in RPE from the control mice were mature (stage IV) at 1 d old (Figure 6A). There was a small reduction in total number of melanosomes at P1 in mutant compared with control RPE, suggesting that any deficiency

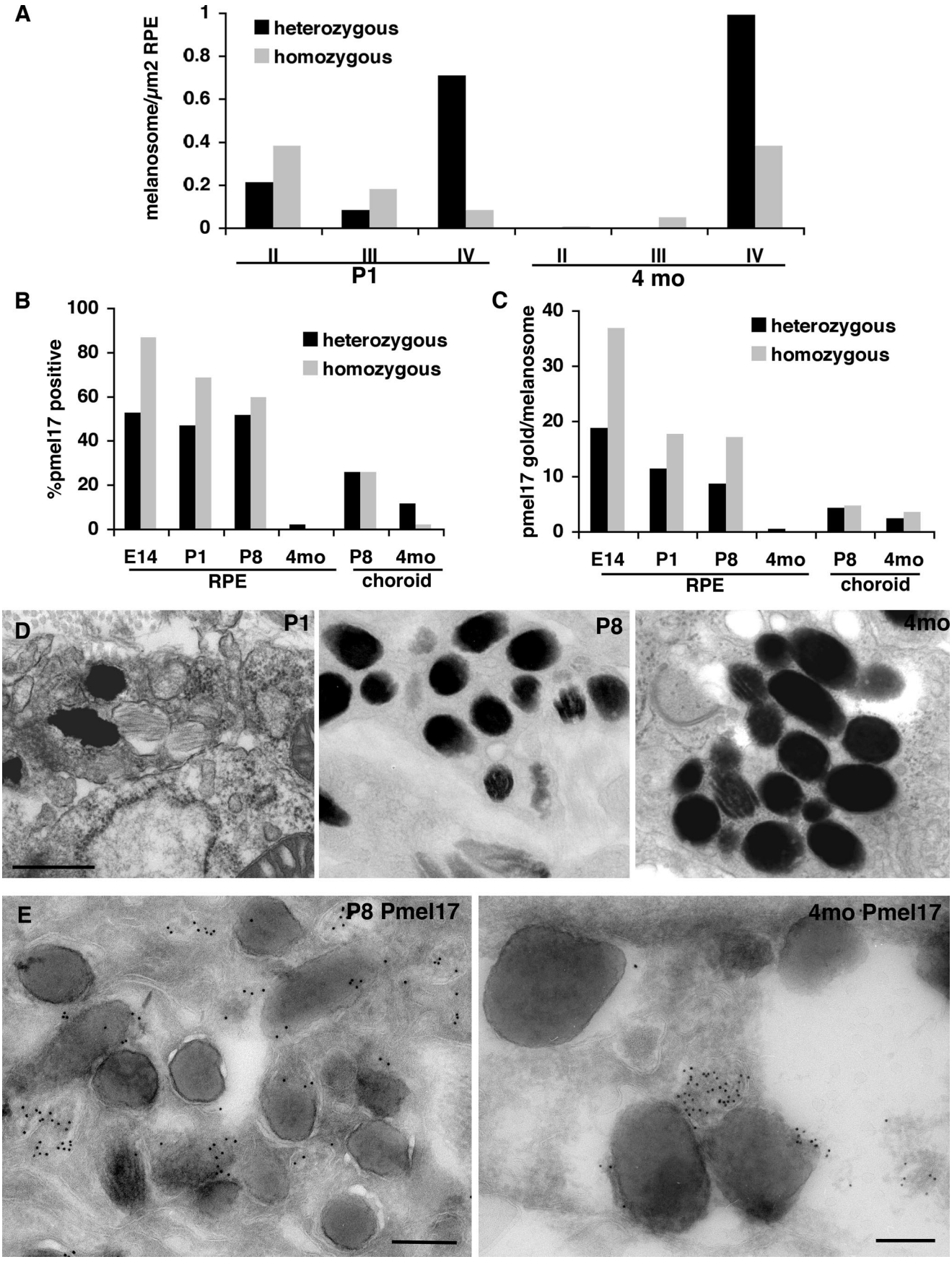

A
melanosome/μm2 RPE
1
0.8
0.6
0.4
0.2
0
heterozygous
homozygous
II
III
IV
P1
II
III
IV
4 mo
B
%pmel17 positive
100
80
60
40
20
0
heterozygous
homozygous
E14
P1
P8
4mo
P8
4mo
RPE
choroid
C
pmel17 gold/melanosome
40
30
20
10
0
heterozygous
homozygous
E14
P1
P8
4mo
P8
4mo
RPE
choroid
D
P1
P8
4mo
E
P8 Pmel17
4mo Pmel17

Figure 6.

in the biogenesis of stage II melanosomes is small. Strikingly, there were very few immature melanosomes in adult homozygous or heterozygous mice (Figure 6A), despite the reduction in the total number of melanosomes in the *cht* RPE (Figures 1 and 6A). It is not possible to determine the precise number of melanosomes that are formed or lost between P1 and adult by counting melanosome number/area of RPE, because the size of the RPE increases during this time. Nevertheless the size of the RPE was approximately the same in heterozygous and homozygous mice and so the apparent loss of melanosomes between P1 and 4 mo in the homozygous, compared with the apparently constant numbers of melanosomes in the heterozygous mice, indicates that immature melanosomes are lost in the mutant *cht* RPE. Quantitation of pmel17 staining in the homozygous RPE showed that there was a higher percentage of pmel17-positive melanosomes than in control RPE at E14, P1, and P8 (Figure 6B), together with more gold particles/melanosome (Figure 6C). The enhanced pmel17 gold labeling in the homozygous *cht* RPE is likely to reflect reduced melanin deposition and hence reduced antigen masking. Therefore, it is clear that the loss of functional Rab38 causes a major reduction in the ability of immature melanosomes to deposit melanin in the RPE, and those that fail to deposit melanin seem to be degraded in the adult mice.

A Low Level of Melanosome Synthesis in Adult cht Choroidal Melanocytes Allows Melanosome Numbers to Recover

Melanocytes of the choroid at P1 contained many immature (stage II) melanosomes in *cht/cht* mice, but by P8 and 4 mo the majority of melanosomes in the choroid were mature (Figure 6D). However, pmel17 staining identified a similar number of immature melanosomes at P8 to that found in control mice (Figure 6, B and E) with similar levels of pmel17 labeling (Figure 6, C and E). Thus, the defect in melanin deposition (and hence antigen masking of pmel17) is less in choroidal melanocytes than in the RPE. Quantitative analysis showed small numbers of pmel17-positive melanosomes (2%) in the adult homozygous choroid, which was less than that observed in heterozygous mice. This could indicate that some immature melanosomes in the *cht/cht* adult choroid fail to deposit melanin and that they are lost even though the continued low levels of melanosome synthesis and tyrosinase delivery allow melanosome numbers to eventually recover in the adult.

Delivery of Tyrosinase to Premelanosomes Is Impaired in the Absence of Rab38

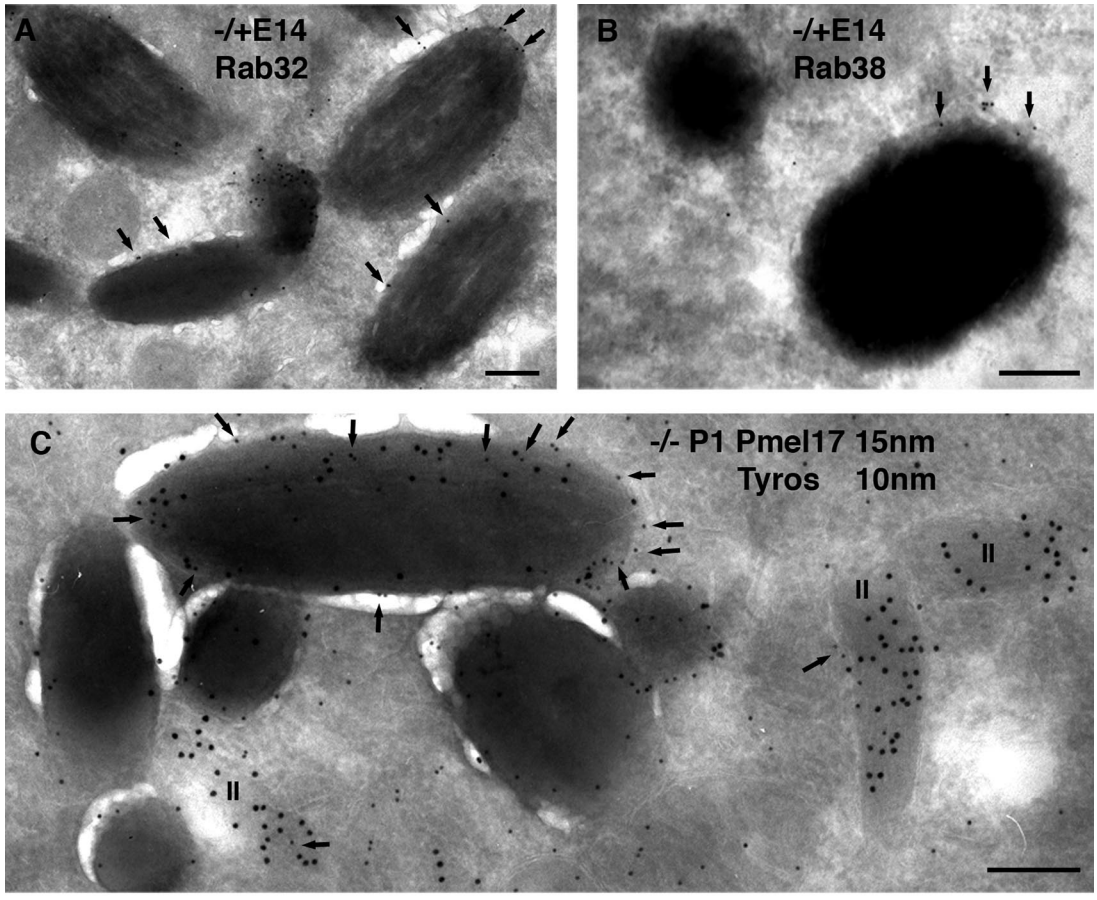
So, what could be the mechanism of action of Rab38 in RPE cells? As a first step toward this goal, we localized endogenous Rab38 and Rab32. Rab32 staining gave a better signal at the cryoimmuno-EM level, and it was detected mainly on the perimeter membrane of mature melanosomes in control E14 RPE (Figure 7A). Low levels of staining could also be observed in the cytoplasm. Rab32 labeling was strongest at E14 and P1, but it declined in the adult (unpublished observations). The signal obtained with anti-Rab38 antibody was lower than with anti-Rab32, but it showed a similar localization (primarily on the perimeter membrane of melanosomes) and a similar expression pattern over time (highest at E14 and low in the adult) (Figure 7B).

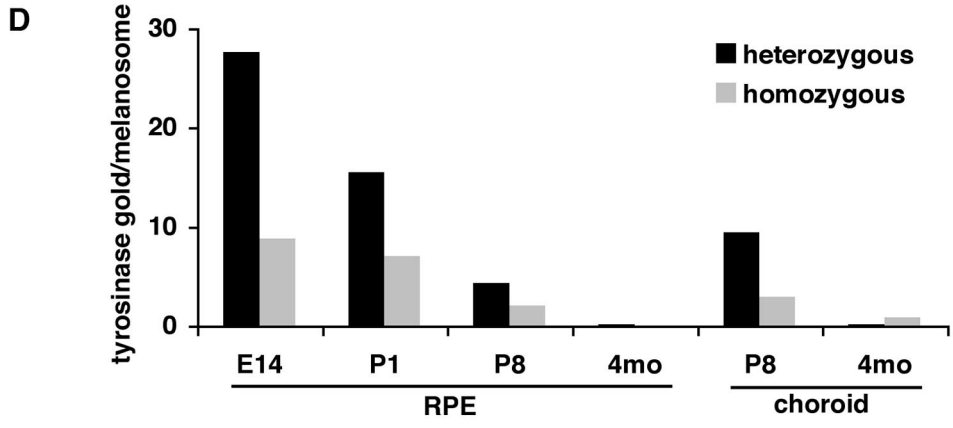
The localization of Rab32/38 to mature melanosomes has also been observed in skin melanocytes (Wasmeier *et al*., 2006), but it is unlikely to represent their only site of action, because they are required for the deposition of melanin in immature melanosomes. A reduction in melanin deposition in melanosomes could reflect an inhibition of delivery of melanin-synthesizing enzymes to the immature melanosome or the environment within the immature melanosome could be unsuitable for their maximum activity. Therefore, the effect of loss of Rab38 on tyrosinase localization was analyzed. RPE cells from P1 mice were cryoimmunolabeled for tyrosinase and pmel17 (to clearly identify immature melanosomes). In *cht/cht* mice, the small number of melanosomes that contained melanin stained strongly for tyrosinase (Figure 7C), as expected, because tyrosinase is required for melanin synthesis. The large numbers of pmel17-positive immature melanosomes contained very little or no tyrosinase staining, suggesting a defect in delivery of tyrosinase to the immature melanosome (Figure 7C). Quantitative analysis of tyrosinase staining (i.e., the mean number of tyrosinase gold particles on both immature and mature melanosomes) showed that the number of tyrosinase gold particles is reduced in both the RPE and the melanocytes of the choroid of the *cht/cht* mice at all time points, compared with heterozygous mice, except in the adult choroid where very few positive melanosomes were available for counting (Figure 7D). The reduction in tyrosinase labeling at P1 in *cht* RPE is greatest on the immature melanosomes, consistent with Figure 7C. However, all time points from E14 to P8 were likewise affected. We noted also some tyrosinase label on tubular and vesicular structures in the cytoplasm, a finding supported by quantitative comparison of relative tyrosinase labeling on melanosomal versus tubulovesicular membranes in *cht* and control RPE at P1. This showed an increase in the relative amount of tyrosinase on tubulovesicular structures in *cht*, compared with control RPE (Figure 7E). Together, these results indicate a defect in the delivery of tyrosinase to immature melanosomes in *cht* cells.

Immature Melanosomes Formed in cht/cht RPE Contain Large Amounts of Cathepsin D

The observation that immature melanosomes were being lost from the RPE to a greater extent in the homozygous mice suggested that immature melanosomes could be degraded in the *cht/cht* mouse. To begin to assess the degradative potential of the maturing melanosomes, homozygous *cht* RPE at P8 was double-stained for cathepsin D and pmel17 to identify immature melanosomes. A small number of cathepsin D-positive vacuoles that lacked pmel17 were presumably lysosomes, although they did not have the electron-dense appearance and multiple lumenal membranes present in lysosomes in many cell types. Another population of vacuoles that contained an approximately equal density of cathepsin D staining colabeled for pmel17, and they were thus identified as immature melanosomes (Figure 8, A and B). Clearly, immature melanosomes that transiently accumulate in the *cht/cht* RPE contain a significant proportion of the

Figure 6 (cont). Premelanosome formation in *cht* choroidal melanocytes. The number of stage II, III, and IV melanosomes per area of RPE (A) in 1-d- and 4-mo-old homozygous and heterozygous *cht* mice was determined from conventional EM images. The percentage of melanosomes (both immature and mature) that were pmel17 positive (at least 5 gold particles/melanosome) in homozygous *cht* mice was quantitated (B), and the mean number of pmel17 gold particles/melanosome (both immature and mature) was determined (C). Data for heterozygous mice is included for comparison. Conventional EM (D) of P1, P8, and 4-mo-old homozygous *cht* choroidal melanocytes and cryoimmuno-EM (E) showing pmel17 staining of homozygous *cht* choroidal melanocytes at P8 and 4 mo old. Pigmented choroidal melanocytes at P1 were too rare to obtain cryoimmuno-EM data. Bars, 0.5 μm (A) and 0.2 μm (B).





E

	Localisation of tyrosinase gold particles (%)	
	Melanosome	Tubulo-vesicular membranes
Heterozygous	91.98	8.02
Homozygous	77.60	22.40

Figure 7. Distribution of Rab32, Rab38, and tyrosinase in the RPE. (A and B) Cryoimmuno-EM showing Rab32 (A) and Rab38 (B) staining of heterozygous *cht* RPE at E14. Bars, 0.2 μm. (C) Cryoimmuno-EM of tyrosinase (10-nm gold; arrows) and pmel17 (15-nm gold) staining of homozygous *cht* P1 RPE. Bar, 0.2 μm. (D) The mean number of tyrosinase gold particles/melanosome (both immature and mature) in homozygous *cht* RPE and choroidal melanocytes was determined. Data for heterozygous mice is included for comparison. (E) The percentage gold particles on melanosomes and on tubulovesicular membranes in RPE at P1 was determined.

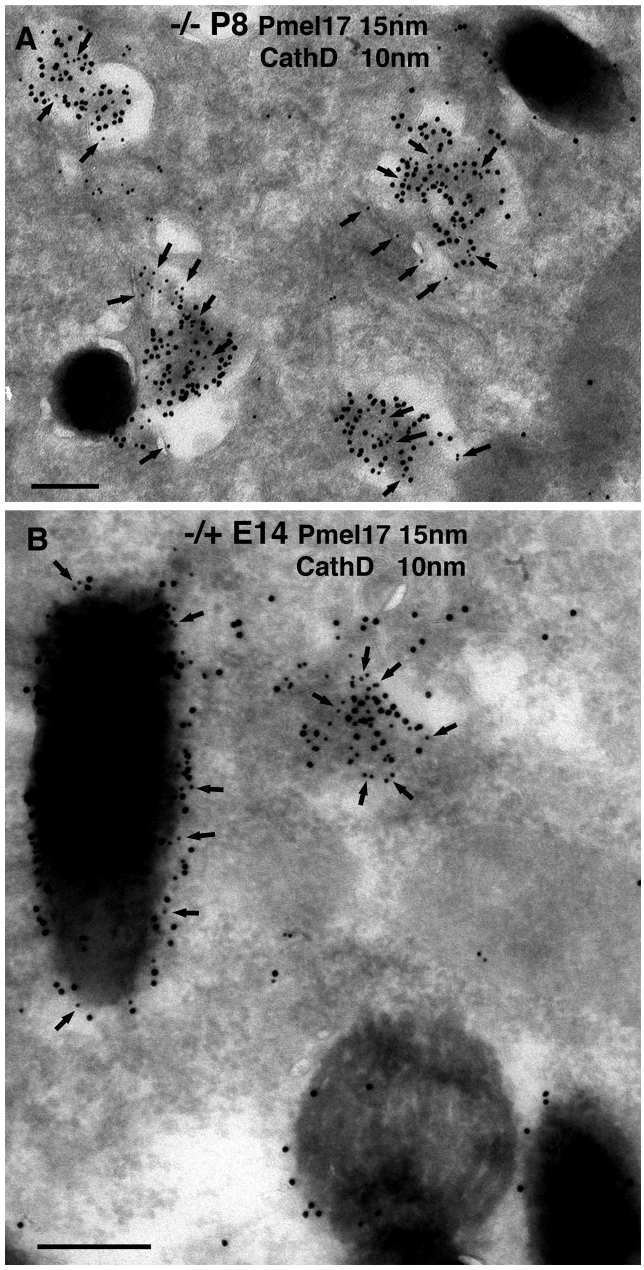


Figure 8. Cathepsin D localization in immature melanosomes in the RPE. Cryoimmuno-EM showing cathepsin D (10-nm gold; arrows) and pmel17 (15-nm gold) staining of homozygous *cht* P8 RPE (A) and heterozygous *cht* E14 RPE (B). Bars, 0.2 μm.

cellular content of cathepsin D, indicating that they may have degradative potential. To determine whether the presence of cathepsin D in immature melanosomes was due to the loss of functional Rab38, double labeling of cathepsin D and pmel17 was performed in control RPE. Because there are very few immature melanosomes at P8, we analyzed E14 RPE and we observed that the immature pmel17-positive melanosomes also contained significant quantities of cathepsin D (Figure 8, A and B).

Premelanosomes Formed in the RPE of albino Mice Lacking Tyrosinase Are Also Unstable

Instability of immature melanosomes in the RPE of *cht/cht* mice could be due to immature melanosomes that fail to deposit melanin being inherently unstable or to a specific loss of function of Rab38. We therefore examined *albino* mice, which fail to deposit melanin in immature melanosomes due to the lack of expression of functional tyrosinase. Although at P1 many immature melanosomes were present in the cytoplasm, as identified by lumenal striations and pmel17 staining, these structures were absent from the adult *albino* mice (Figure 9). Strikingly, there was no detectable pmel17 staining (Figure 9), confirming that, as observed in *cht* mice, the immature melanosomes that fail to deposit melanin seem to be unstable.

As described above, melanosome production in the melanocytes of the choroid begins later than in the RPE and extends after birth, being present at a low rate during adulthood. Choroidal melanocytes of the homozygous *cht* mouse have immature melanosomes, which could be readily identified by pmel17 staining despite the prevalence of mature melanosomes (Figure 6). In adult *albino* mice, we could observe many immature melanosomes in choroidal melanocytes, a finding that was very clear given the absence of mature melanosomes (Figure 9).

DISCUSSION

Loss of functional Rab38 or Rab32 in melanocytes of the skin has little effect on pigmentation, but loss of both Rab proteins causes dramatic depigmentation (Wasmeier *et al*., 2006). This suggests that Rab38 and the 67% homologous Rab32 are at least partially functionally redundant in skin melanocytes. Here, we show that loss of functional Rab38 alone causes a dramatic reduction in melanosome number within adult RPE cells. This suggests that Rab32 is unable to compensate for loss of functional Rab38 in the RPE. Rab32 is readily detectable in the RPE, so the failure of Rab32 to compensate for loss of Rab38 in the RPE is not due to lack of expression. We suggest that the short time window during which melanosomes are formed in the RPE renders the RPE very sensitive to decreases in the efficiency of melanosome biogenesis.

The Timing of Melanosome Formation in Ocular Tissues

Although the timing of melanin synthesis has been extensively studied in the RPE, the biogenesis of the melanosome has been less well characterized. Melanin synthesis begins in the embryonic RPE, but controversy remains over whether there is a low level of melanin synthesis in the adult RPE. Several studies have reported an absence of tyrosinase and/or tyrosinase-related protein expression in adult RPE (Miyamoto and Fitzpatrick, 1957; Smith-Thomas *et al*., 1996). However, tyrosinase activity has been detected in extracts from adult RPE (Dryja *et al*., 1978; Varela *et al*., 1995), and tyrosinase expression/activity was detected in cultured adult RPE cells (Basu *et al*., 1983; Dorey *et al*., 1990), and it was increased after incubation with rod outer segments (Schraermeyer *et al*., 2006). Vacuoles with the morphological characteristics of immature melanosomes have been described in adult RPE (Schraermeyer, 1993; Schraermeyer and Heimann, 1999), but pmel17 staining in adult RPE has not been reported. We were unable to detect tyrosinase expression in adult mouse RPE by cryoimmuno-EM. Levels of expression were high in E14 eyes and P1, but they were much lower by P8. Although the comparatively low sensitivity of cryoimmuno-EM does not preclude low levels of enzyme expression in the adult, it is clear that the majority of melanin synthesis occurs in the embryonic RPE. Pmel17 staining was found in E14 RPE on vacuoles containing discrete internal vesicles, on vacuoles containing striations, and

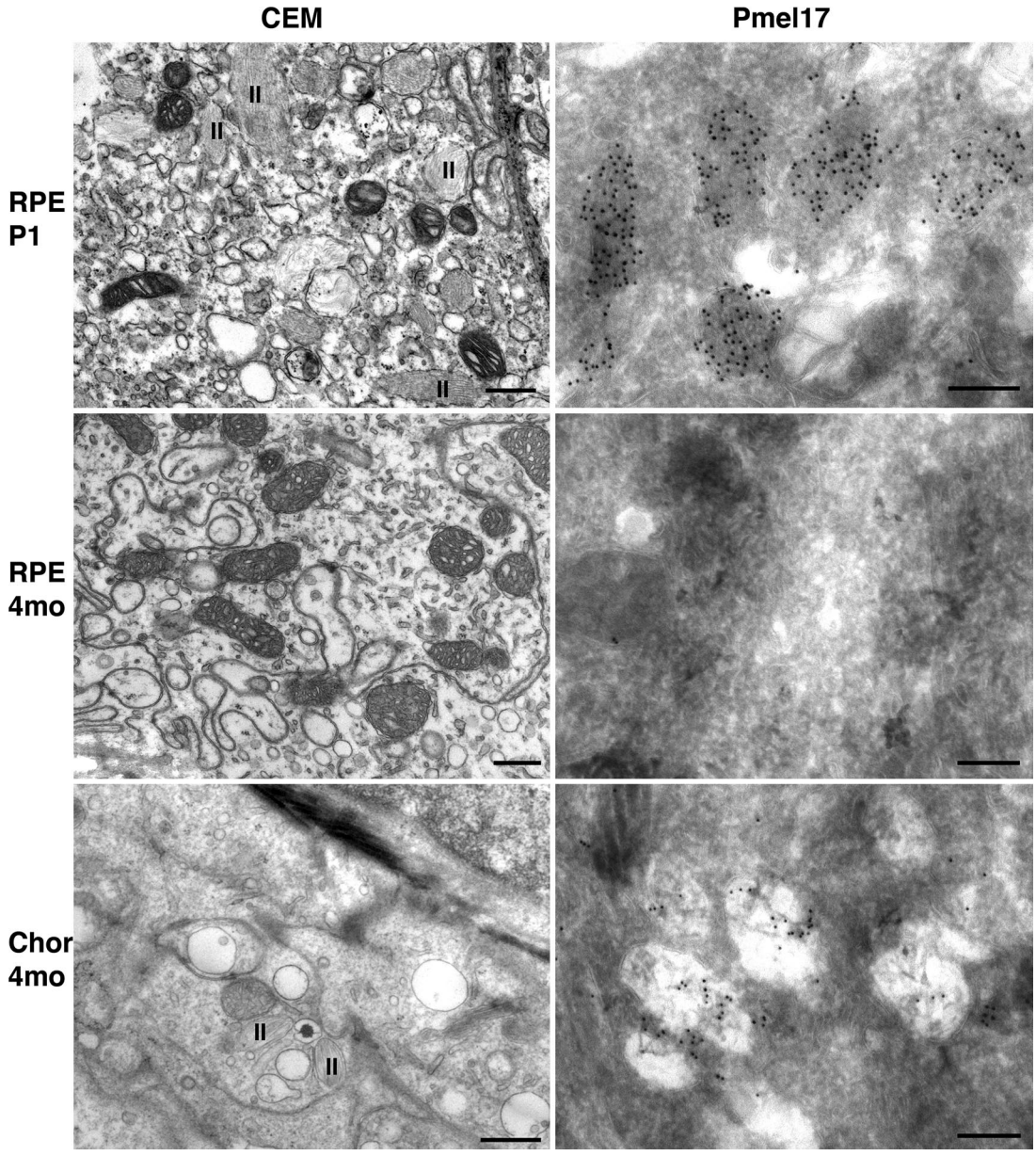


Figure 9. Premelanosome formation in *albino* RPE and choroidal melanocytes. Conventional EM and cryoimmuno-EM showing pmel17 staining of albino RPE and choroidal melanocytes (chor) at P1 and 4 mo. Bars for conventional EM, 0.5 μm and for cryoimmuno-EM, 0.2 μm.

on vacuoles containing striations upon which melanin was deposited and thus was found on vacuoles with the morphological characteristics of stage I, II, and III melanosomes, as described by Raposo *et al*. (2001) in cultured skin melanocytes. Unlike skin melanocytes (Raposo *et al*., 2001) and choroidal melanocytes (this study), mature melanosomes in RPE cells retained some staining for pmel17 at early time points. Possibly, this could reflect differences in the structural pattern of melanin deposition in melanosomes of RPE cells. We could not find pmel17-staining vacuoles in adult RPE; so, if there is a low level of melanin synthesis in the adult, this could reflect replenishment of the melanin content of existing melanosomes, rather than synthesis of new melanosomes.

There are few reported studies on melanin turnover and melanosome biogenesis in melanocytes of the choroid, although tyrosinase activity has been reported in extracts from adult choroid (Nakazawa *et al*., 1985; Varela *et al*., 1995). Melanocytes could not be identified in the choroid in heterozygous *cht* E14 mice, but by P1 choroidal melanocytes contained both immature and mature melanosomes, suggesting that most melanosome production occurs after birth. Pmel17 and melanin-synthesizing enzymes were still readily detectable in the choroid at a time (P8) when their expression in the RPE had declined. In the adult choroid, their expression was low but still detectable. Thus, melanosome biogenesis is sustained for a longer period in choroidal melanocytes than in the RPE, but it is not constant throughout life. This is consistent with the hypothesis that the length of the time window for melanosome production determines the severity of the phenotype in the *cht* mouse because the choroidal melanocytes are less affected than the RPE but more affected than skin melanocytes. That a low level of synthesis of melanosomes occurs throughout life in choroi-

dal melanocytes is demonstrated by the increased numbers of melanosomes in choroidal melanocytes at 2 y old, compared with 4 mo old, in all mice examined. This continued synthesis allowed melanosome numbers to largely recover to wild-type levels in choroidal melanocytes of older *cht* mice.

Rab38 Is Not Required for the Biogenesis of Immature Melanosomes, but It Is Required for the Delivery of Melanin-synthesizing Enzymes to Them

Analysis of the RPE of adult *cht* mice showed little or no immature melanosomes. The small number of melanosomes present seemed fully melanized and showed a normal distribution, in that they had access to the apical processes of the RPE. This at first suggested a defect in the biogenesis of immature melanosomes. However analysis of P1 RPE from *cht* mice, a time point when melanogenesis is largely complete, showed that areas of cytoplasm were packed with stage II melanosomes, and a quantitative comparison of melanosome numbers between control and *cht* RPE indicated that there was little defect in immature melanosome biogenesis.

The large number of immature melanosomes that are present at P1 and P8 contain very little tyrosinase, indicating that there is a defect in the delivery of tyrosinase to the immature melanosome in the *cht* eye. The small number of mature melanosomes that deposit melanin in the *cht* mouse stain for tyrosinase (as expected, because tyrosinase is the rate-limiting enzyme for melanin synthesis), albeit with less intensity than mature melanosomes of the control RPE.

AP3 or BLOC-1 deficiency in skin melanocytes results in accumulation of tyrosinase in endosomes (Theos *et al*., 2005; Setty *et al*., 2007), showing that these protein complexes function in transfer of tyrosinase from endosomes to melanosomes. No clear endosomal accumulation was observed in melanocytes of the skin (Wasmeier *et al*., 2006) or in the RPE (this study) from Rab38-deficient mice. There was an increase in the ratio of tyrosinase labeling on tubulovesicular membranes versus melanosomes in *cht* mice, compared with heterozygous controls, but no clear accumulation in a readily identifiable membranous compartment within the cell. One possible explanation for these observations is that in the absence of Rab38, these vesicles are delivered to the lysosome and degraded. A similar conclusion was reached in skin melanocytes lacking both Rab38 and Rab32 where inhibition of new protein synthesis caused tyrosinase labeling to be lost from the cells (Wasmeier *et al*., 2006).

The Fate of Melanosomes in the RPE

Because few melanosomes are formed after birth in the RPE, it was possible to follow a single cohort of melanosomes. Both *albino* and *cht* RPE fail to deposit melanin within melanosomes, in *albino* because tyrosinase is not expressed, and in *cht* because tyrosinase fails to be delivered. Consistent with previous reports, immature melanosomes are made in both cases, but we have made the novel observation that immature melanosomes that fail to deposit melanin in the RPE are lost in the adult. Loss of immature melanosomes could be due to fusion with the lysosome, the immature melanosome itself could have degradative activity, immature melanosomes could be secreted or they could be subject to autophagy. Melanosomes of RPE cells have previously been reported to contain acid phosphatase (Feeney, 1978) and cathepsin D (Azarian *et al*., 2006). Mature melanosomes of skin melanocytes also have been reported to contain acid hydrolase activity and lysosomal membrane proteins (Orlow, 1995), although quantitative cryoimmuno-EM found that the majority of cathepsin D or lysosomal markers was in lysosomes, rather than melanosomes in skin melanocytes (Raposo *et al*., 2001). We found high concentrations of cathepsin D in the immature melanosomes that accumulate in early postnatal *cht* RPE. The presence of similar concentrations of cathepsin D in immature melanosomes from E14 RPE from heterozygous mice suggests that during melanosome biogenesis cathepsin D is delivered to the maturing melanosome. It is possible that the rapid deposition of melanin within the maturing melanosome prevents the autodegradation of the organelle, but in the *cht* and *albino* mice the absence of this protection causes the immature melanosomes to gradually degrade themselves. However, we cannot exclude the possibility that the other mechanisms described above could contribute to the loss of immature melanosomes that fail to deposit melanin.

Tyrosinase and Tyrp1 are markers of mature melanosomes in skin melanocytes (Raposo *et al*., 2001). In contrast, in melanosomes of RPE cells tyrosinase rapidly disappears from the melanosome after melanin deposition, because tyrosinase levels were considerably reduced on many melanosomes by 8 d and undetectable in the adult. This implies that either tyrosinase in the mature melanosome is degraded in situ by lysosomal enzymes within the melanosome or that it is removed from the mature melanosome after melanin synthesis is complete and it is delivered to the lysosome and degraded. A membrane remodeling process has been described for the maturation of secretory granules during which lysosomal enzymes are removed from the maturing granule by mechanisms involving AP1, clathrin, and GGAs (Dittie *et al*., 1997; Klumperman *et al*., 1998; Kakhlon *et al*., 2006). Such a process whereby tyrosinase is removed from the mature melanosome has not been described in studies of skin melanocytes, but those melanosomes are released to keratinocytes after melanin deposition. Tyrosinase has a dileucine-based signal that is recognized by AP3 (Honing *et al*., 1998), and both AP1 and AP3 function in the delivery of tyrosinase from the endosome to the melanosome in skin melanocytes (Theos *et al*., 2005). Whether these adaptors can also play a role in transfer of tyrosinase from the melanosome to the lysosome is unknown, although the destination of tyrosinase when expressed in nonmelanogenic cells is the lysosome, indicating that the dileucine signal may mediate lysosomal targeting (Calvo *et al*., 1999; Simmen *et al*., 1999).

Melanosome Biogenesis in RPE Cells versus Melanocytes

The results of this study highlight both parallels and differences in melanosome biogenesis between melanocytes and RPE cells. Melanosome biogenesis in vivo in the RPE proceeds through the four stages of melanosome maturation that have been elegantly described in cultured skin melanocytes (Seiji and Iwashita, 1963; Raposo *et al*., 2001). Rab38 is required for the efficient delivery of melanin-synthesizing enzymes to the maturing melanosome in both skin melanocytes and RPE cells. Melanosomes within the RPE that fail to deposit melanin are unstable and they are degraded. In RPE cells, "mature" melanosomes rapidly lose melanin-synthesizing enzymes, indicating that further maturation events take place after melanin deposition. Possibly, this is an adaptation that occurs in RPE cells that is related to the long life of the melanosome.

The pathways that lead to the biogenesis of melanosomes and other lysosome-related organelles are complex. Insight into these pathways has been gained from studies of mouse coat color mutants defective in different components of these pathways. Like the *cht* mouse, mutations in many of these components do not lead to an absence of melanosomes,

indicating that there is some redundancy in these pathways. The results of the present study show that the RPE is exquisitely sensitive to defects in biogenetic pathways, and they provide an opportunity to follow the biogenesis, maturation, and fate of a single cohort of melanosomes in a way not possible in skin melanocytes. A comparison of the phenotype of different mutations at time points during melanosome biogenesis and maturation in the RPE may yield novel information on the regulation of the biogenesis not only of melanosomes but also of other lysosome-related organelles.

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